Example 8: Immunization with pCWNVCp Induces Potent Antigen-Specific Th1-Type Cellular Immune Responses.

The level of cytokines released by T cells reflects the direction and magnitude of the immune response. The level of Th1 (IFN-γ and IL-2) and Th2 (IL-4) type cytokines produced by stimulated T cells were examined. IFN-γ, a prototypical Th1-type cytokine, is produced predominantly by CD4+ Th1 cells and CD8+ T cells. The level of IFN-γ expressed by stimulated T cells reflects the magnitude of the T cell response. IL-2 is a Th1-type cytokine produced primarily by T cells activated by external stimulation; it is critical for the proliferation and clonal expansion of antigen-specific T cells (Morgan *et al.*, 1976, Selective *in vitro* growth of T lymphocytes from normal human bone marrows, Science, 193:1007-1008, which is incorporated herein by reference). On the other hand, IL-4 is a prototypical Th2-type cytokine that plays a dominant role in B cell-mediated immune responses (Seder & Paul, 1994, Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annu. Rev. Immunol., 12:635-673, which is incorporated herein by reference).

The level of CD4+ T helper cell-mediated immune responses following immunization was also examined. Mice received two DNA immunizations (100 μg each) separated by two weeks. At one week after the second injection, the mice were euthanized, the spleens harvested. Lymphocytes were harvested from spleens and prepared as effector cells by removing the erythrocytes and by washing several times with fresh media as described in Kim *et al.*, 1997, Engineering of in vivo immune responses to DNA immunization via co-delivery of costimulatory molecule genes, Nat. Biotechnol., 15:641-646, which is incorporated herein by reference. The isolated cell suspensions were resuspended to a concentration of 5×10^6 cells/ml. A 100 μl aliquot containing 5×10^5 cells was immediately added to each well of a 96 well microtiter flat bottom plate. WNV capsid-specific peptide pools (WNVC-P1: SKKPGGPGKSRAVNMLKRGMPR; WNVC-P2: KRAMLSLIDGKGPIRFVLA; WNVC-P3: TLTSAINRRSSKQKKRGGKTGI) at the final concentration of 5 μg/ml were added to wells in triplicate. The cells were incubated at 37°C in 5% CO_2 for 4 days. Supernatants from these wells were collected at day 4 and tested for tested for the release of IFN-γ, IL-2, or IL-4 by cytokine ELISA using ELISA kits (Biosource, Camarillo, CA; R&D Systems, Minneapolis, MN).

As shown in Figure 13, significant expression levels of IFN-γ and IL-2 were observed from pCWNVCp-immunized mice, while only background levels were observed from control-

immunized mice. On the other hand, the level of IL-4 released from all immunized groups was similar. These results show that DNA vaccination resulted in induction of specific and potent Th1-type cellular immune responses in immunized mice.

Example 9: Immunization with pCWNVCp Induces Antigen-Specific Production of Chemokines MIP-1β and RANTES.

The characterization of vaccine-induced cellular immune reponses was extended by examining the expression profiles of β-chemokines (MIP-1β and RANTES) from stimulated T cells. Chemokines are important modulators of immune and inflammatory responses. They are especially important in the molecular regulation of trafficking of leukocytes from the vessels to the peripheral sites of host defense. T cell-produced chemokines have been reported to play a critical role in cellular immune expansion (Kim *et al.*, 1998, J. Clin. Invest., *supra*; Kim *et al.*, 2000, Macrophage-colony stimulating factor (M-CSF) can modulate immune responses and attract dendritic cells in vivo, Human Gene Therapy, 11:305-321,which is incorporated herein by reference). Therefore, the level of chemokines produced by stimulated T cells may provide additional insight on the level and the quality of antigen-specific cellular immune responses. Supernatant from the T cells stimulated as described in Example 8 was tested for the release of β-chemokines MIP-1β and RANTES using ELISA kits (Biosource, Camarillo, CA; R&D Systems, Minneapolis, MN). Immunization with pCWNVCp vaccine induced significantly greater levels of expression of MIP-1β and RANTES over those of control vector immunization (Figure 14). These increased levels of MIP-1β and RANTES from pCWNVCp immunized animals further support the conclusion that pCWNVCp immunization induced the antigen-specific T cell responses observed above.

Example 10: Immunization with pCWNVCp Induces an Antigen-Specific CTL Response.

The level of antigen-specific cytotoxic T lymphocyte (CTL) responses following immunization was also examined. Afive hour ^{51}Cr release bulk CTL assay was performed, as previously described Kim *et al.*, 1997, Nat. Biotechnol., *supra*, with *in vitro* stimulation of effector splenocytes prior to measuring chromium release from specific and non-specific peptide treated targets. Effector splenocytes were stimulated *in vitro* with a pool of WNV Capsid peptides (KGPIRFVL (SEQ ID NO:24), GGPGKSRA (SEQ ID NO:25), and IAPTRAVL (SEQ ID NO:26)) at a concentration of 10 μg/ml for five days in CTL culture media at 5 x 10^6 cells/ml.

CTL culture media consists of RPMI 1640 (Gibco-BRL, Grand Island, NY), 10% fetal calf serum (Gibco-BRL) and 10% RAT-T-STIM without Con A (Becton Dickinson Labware, Bedford, MA). Peptide treated targets were prepared by incubating P815 mouse mastocytoma cells (ATCC, Manassas, VA) with 10 μg/ml concentration of the peptide pool. The target cells were labeled with 100 μCi/ml $Na_2{}^{51}CrO_4$ for 120 minutes and incubated with the stimulated effector splenocytes for six hours at 37°C. CTL lysis was determined at 100:1 and 50:1 effector:target (E:T) ratios. Percent specific lysis was determined from the formula:

100 x (experimental release - spontaneous release) / (maximum release - spontaneous release)

Maximum release was determined by lysis of target cells in 1% Triton X-100 containing medium. An assay was not considered valid if the value for the 'spontaneous release' counts were in excess of 20% of the 'maximum release.' A background level of specific killing was observed from the control animals immunized with pCDNA3. However, the animals immunized with pCWNVCp showed positive CTL activities at 100:1 and 50:1 effector to target (E:T) ratios (Figure 15A). In addition, an analysis of the supernatant from the *in vitro* stimulated effector cells for the CTL assay demonstrated an increased level of IFN-γ production from pCWNCp-immunized mice (Figure 15B).

Example 11: Immunization with pCWNVCp Induces Infiltration of Lymphocytes into the Muscle of Immunized Animals.

The magnitude of vaccine-induced cell-mediated immune responses in HIV and HSV DNA immunization models has been found to correlate well with the level of cellular infiltration at the site of vaccine injection (Kim *et al.*, 2000, Human Gene Therapy, *supra*; Chattergoon *et al.*, 2000, Targeted antigen delivery to antigen-presenting cells including dendritic cells by engineered Fas-mediated apoptosis, Nat. Biotechnol., 18:974-979, which is incorporated herein by reference; Agadjanyan *et al.*, 1999, CD86 (B7-2) can function to drive MHC-restricted antigen-specific cytotoxic T lymphocyte responses *in vivo*., J. Immunol., 162:3417-3427, which is incorporated herein by reference). To further investigate the potency of immune activation induced by pCWNVCp immunization, the muscle tissues of immunized mice were examined immunohistochemically at the site of injection.